The *qSD12* underlying gene promotes abscisic acid accumulation in early developing seeds to induce primary dormancy in rice

Xing-You Gu · Tianlei Liu · Jiuhuan Feng ·
Jeffrey C. Suttle · James Gibbons

Abstract Seeds acquire primary dormancy during their development and the phytohormone abscisic acid (ABA) is known to play a role in inducing the dormancy. *qSD12* is a major seed dormancy quantitative trait locus (QTL) identified from weedy rice. This research was conducted to identify *qSD12* candidate genes, isolate the candidates from weedy rice, and determine the relation of the dormancy gene to ABA. A fine mapping experiment, followed by marker-assisted progeny testing for selected recombinants, narrowed down *qSD12* to a genomic region of <75 kb, where there are nine predicted genes including a cluster of six transposon/retrotransposon protein genes and three putative (a PIL5, a hypothetic protein, and a bHLH transcription factor) genes based on the annotated Nipponbare genome sequence. The PIL5 and bHLH genes are more likely to be the QTL candidate genes. A bacterial artificial chromosome (BAC) library equivalent to 8–9 times of the haploid genome size was constructed for the weedy rice. One of the two BAC contigs developed from the library covers the PIL5 to bHLH interval. A pair of lines different only in the QTL-containing region of <200 kb was developed as isogenic lines for the *qSD12* dormancy and non-dormancy alleles. The dormant line accumulated much higher ABA in 10-day developing seeds than the non-dormant line. In the QTL-containing region there is no predicted gene that has been assigned to ABA biosynthetic or metabolic pathways. Thus, it is concluded that the *qSD12* underlying gene promotes ABA accumulation in early developing seeds to induce primary seed dormancy.

Keywords Seed dormancy · Quantitative trait locus · Fine mapping · Abscisic acid · Rice

Introduction

Seeds acquire primary dormancy during their development to maintain an arrest status after maturation for varying periods depending on genotypes and environmental conditions. It is the genotypic variation that provides wild species with an adaptive mechanism to survive diverse environments and also provides plant breeders with raw materials to manipulate germinability of crop varieties. Currently, many quantitative trait loci (QTL) have been associated with the genotypic variation in cereal crops and some other plants. A confirmed QTL refers to a marked genomic region usually ranging from a few to >10 centi-Morgan (cM) in genetic distance. In rice (*Oryza sativa* L.), such a region may consist of >100 genes based on the reference genome sequence (IRGSP 2005). In addition, a dormancy QTL itself does not infer any physiological/biochemical functions related to seed development or germination. Thus, the reported QTL remain to be characterized for underlying genes and physiological functions.

Phytohormone abscisic acid (ABA) is a major signaling molecule involved in induction of seed dormancy (Finch-Savage and Leubner-Metzger 2006; Finkelstein et al. 2008;

X.-Y. Gu (✉) · T. Liu · J. Feng
Plant Science Department, South Dakota State University,
Brookings, SD 57007, USA
e-mail: Xingyou.gu@sdstate.edu

J. C. Suttle
Northern Crop Science Laboratory, USDA-ARS, Fargo,
ND 58105, USA

J. Gibbons
Rice Research and Extension Center, University of Arkansas,
Stuttgart, AR 72160, USA

Holdsworth et al. 2008). Evidence for this function was mainly collected from artificial mutants deficient in or insensitive to ABA (e.g., Karssen et al. 1983; Lefebvre et al. 2006; Fang et al. 2008). Research done in Arabidopsis suggested that ABA synthesized in embryo and endosperm tissue is more important than that synthesized in maternal tissues in inhibiting germination (Lefebvre et al. 2006). The reported QTL represent naturally occurring genes regulating variation in primary seed dormancy. Map-based cloning of the Arabidopsis *DOG1* seed dormancy QTL identified an underlying gene with unknown molecular function (Bentsink et al. 2006). The remaining QTL are yet to be determined for their relations to ABA biosynthesis or metabolism.

The *qSD12* locus is one of the seed dormancy QTL identified from a line of tropical weedy rice (*O. sativa*) and has the largest phenotypic effect on inhibiting germination (Gu et al. 2004). However, this major QTL has not been reported for cultivated and wild (including *O. rufipogan* and *nivara*) rice (Lin et al. 1998; Cai and Morishima 2000; Thomson et al. 2003; Lee et al. 2005; Li et al. 2006). Later research suggested that *qSD12* has potential to be used to breed varieties resistant to pre-harvest sprouting as it does not associate with other undesirable weedy characters (Gu et al. 2005), and that the locus holds important information on regulatory mechanisms of seed dormancy because it interacts with other QTL and with the seed development environment and expresses in embryo and/or endosperm tissue (Gu et al. 2006, 2008). The objectives of this research were: (1) to fine map the *qSD12* region to narrow its candidate genes; (2) to develop a bacterial artificial chromosome (BAC) library for weedy rice to isolate the *qSD12* dormancy allele; and (3) to determine the effect of *qSD12* on ABA in developing seeds in an isogenic background to better understand regulatory mechanism of this major seed dormancy gene.

Materials and methods

Plant materials and cultivation

The previous research introduced a single chromosome segment of about 600 kilo bases (kb) containing the *qSD12* dormancy allele from the weedy rice line SS18-2 into the genetic background of the cultivated rice line EM93-1 by generations of recurrent backcrossing and marker-assisted selection (Gu et al. 2005, 2008). Seeds from the plants heterozygous only for this introgression segment were germinated in an incubator at 30°C. Seedlings were planted in rice culture solution (Yoshida et al. 1976) in the greenhouse to identify recombinants between markers on the target region. Recombinant plants were transplanted into pots filled with solid medium to harvest seeds for progeny testing.

For progeny testing, seeds from each recombinant plant were left at room temperature for about 30 days (i.e., warm, dry after-ripening) to beak dormancy prior to germination. Seedlings were grown in 13-cM pots (one plant per pot) filled with solid medium in the greenhouse, or transplanted in the field with one plant per hill at the Rice Research and Extension Center, University of Arkansas, Stuttgart, Arkansas, USA. Seeds were harvested from individual plants at 40 days after flowering and then air-dried for 3 days. The dried seeds from greenhouse or field plants were stored in a −20°C freezer or a 5°C cold room to maintain the dormant status.

Seed dormancy assessment

Seed dormancy was quantified by germination percentage. Prior to germination, seed samples were maintained at about 25°C for 1–3 weeks (i.e., partial after-ripening) to better display genotypic differences in seed dormancy. Fifty seeds for each of three replications were placed in a 9-cm Petri dish lined with Whatman No. 1 filter paper, wetted with 10 ml of de-ionized water, and then incubated at 30°C and 100% relative humidity in the dark. Germination was evaluated visually by protrusion of the radicle or coleoptile from the hull by ≥ 3 mm at day 7 or daily for a period of 10 days, depending on the experimental purpose.

Marker development and genotyping

New PCR-based DNA markers were developed by searching for simple sequence repeats (SSR) from the Nipponbare genome sequence (IRGSP 2005) corresponding to the *qSD12* region. PCR primers were designed using Primer3 software (Rozen and Skaletsky 2000). For marker genotyping, genomic DNA was prepared from fresh leaf tissue of the greenhouse grown plants or a bulk sample of bud tissue from >30 germinated seeds from the individual field grown plants. DNA extraction, PCR, and gel electrophoresis for marker display were conducted using previously described methods (Gu et al. 2004). The markers polymorphic between EM93-1 and SS18-2 (Table 1) were used to genotype individual plants from the mapping population or progeny lines and to delimit the SS18-2-derived sub-segments retained in recombinant plants.

BAC library construction and BAC clone screening and contig development

A BAC library for SS18-2 was constructed to isolate the dormancy alleles at *qSD12* and other QTL from weedy rice. About 2-week-old seedlings were kept in the dark for about 24 h to harvest leaf tissue for magabase size DNA preparation. The library was constructed using the

Table 1 Summary of information on newly developed simple sequence repeat (SSR) markers polymorphic between the parental lines EM93-1 and SS18-2

Marker name	Forward primer	Reverse primer	SSR motif	Position (bp)[a]	Expected size (bp)
SD12m05	catcaaccggtgagaaaggt	ggagaccgtgctcttatcca	(GA)6	25179741	113
SD12m10	gccaaacagggtgaaagaga	catcctccatgcatcgatct	(GA)9	25185591	124
SD12m13	tgcaaacgagaagaagagaga	gcactgagcatgatgtggat	(AG)7	25191778	136
SD12m43	gaaccgctgaaacccaataa	ggcaacaatgtcctgctttt	(AAC)5	25173535	98
SD12m45	tatcagtcgaccgtgaggtg	tatatagggcgtgggcatga	(GAC)6	25175285	105
SD12m50	gccgtccagcttcttcttc	gaggcttaagacccaccctta	(TCT)6	25196809	115
SD12m62	gtccgtcctgtagccattgt	taccaacacgagctcaccaa	(AGG)10	25238219	81

[a] Physical position on rice chromosome 12

restriction enzyme *Bsty*I at Clemson University Genomics Institute. The resulting library consists of 36,864 clones, which were double-gridded on two 22 × 22-cm GE HealthCare Hybond N⁺ membranes in a 4 × 4 format.

The BAC library was screened using an overlapping oligonucleotide (Overgo) hybridization technique (Song et al. 2005) to identify clones containing *qSD12* candidate genes from the weedy rice donor. Overgo primers were designed based on single copy segments of 40 (37–44) bp selected from the Nipponbare (IRGSP 2005) and 93-11 genome sequences (http://www.ncbi.nlm.nih.gov). A total of 20 Overgos, relatively evenly distributed on the 417 (from 24,942 to 25,359)-kb interval on chromosome 12, were selected to cover the narrowed *qSD12* and its flanking regions. The primers labeled with [α-^{32}P] dATP and [α-^{32}P] dCTP were hybridized with membrane filters following the protocol at https://www.genome.clemson.edu/protocols.shtml. The filters were washed twice using 1× SSC and 0.1% SDS before exposure to X-ray film to display positive spots. Positive clones were purified and confirmed by PCR for 19 microsatellite markers or Overgo-containing segments between RM28607 and RM28692.

Confirmed BAC clones were end-sequenced for about 500 bp. End sequences were used as queries to BLAST against the Nipponbare genome sequence from Gramene data base (http://www.gramene.org/Oryza_sativa/index.html) to position the clones and to estimate their sizes. A BAC contig was developed based on the physical positions. This hybridization experiment was repeated to confirm the gap between two BAC contigs developed in the initial experiment.

Isogenic line development and ABA detection

Plants homozygous for the SS18-2- or EM93-1-derived alleles were selected from the recombinant #0315-derived progeny line based on marker genotypes. Self-pollinated seeds from the selected plants were used as isogenic lines for the *qSD12* dormancy (IL$_{SD12}^{DD}$) and non-dormancy (IL$_{SD12}^{dd}$) alleles, respectively. The two isogenic lines were grown in a greenhouse and plants that flowered on the same day were used to harvest intact seeds at 0 (spikelet), 10, 20, 30, and 40 days after anthesis. Seed samples of 2 g each were collected in liquid nitrogen and then stored in a −80°C freezer. Sample preparation and ABA tests were conducted using previously described methods (Destefano-Beltrán et al. 2006).

Statistic analyses

Germination percentage (*y*) for a sample was transformed by $\sin^{-1}(y)^{-0.5}$ and transformed values were averaged over three replications for a progeny plant. According to the previous research, when genic effects of *qSD12* on germination were evaluated based on maternal genotypes, only the additive component was significant (Gu et al. 2008). Therefore, germination data were correlated with genotypes of the marker segregating in the progeny lines to determine if the *qSD12* dormancy allele was retained in the recombinant plant. A significant correlation (*r*) indicates that the dormancy gene locates on the heterozygous region in the recombinant (Fig. 1b). A non-significant *r* value could be the result of the dormancy gene locating on the genomic region homozygous for the EM93-1 or SS18-2-derived chromosomal segment in the recombinant (Fig. 1b). The linear correlation analysis was performed by the SAS CORR procedure (SAS Institute 1999).

Results

Fine mapping of the *qSD12* region

Marker genotyping of 3,600 plants identified 34 recombinant events between RM28607 and RM28652. The recombination fraction for the genomic region of about 500 kb was estimated as 0.47% (i.e., 34 recombinant events from 7,200 gametes). These recombinant plants
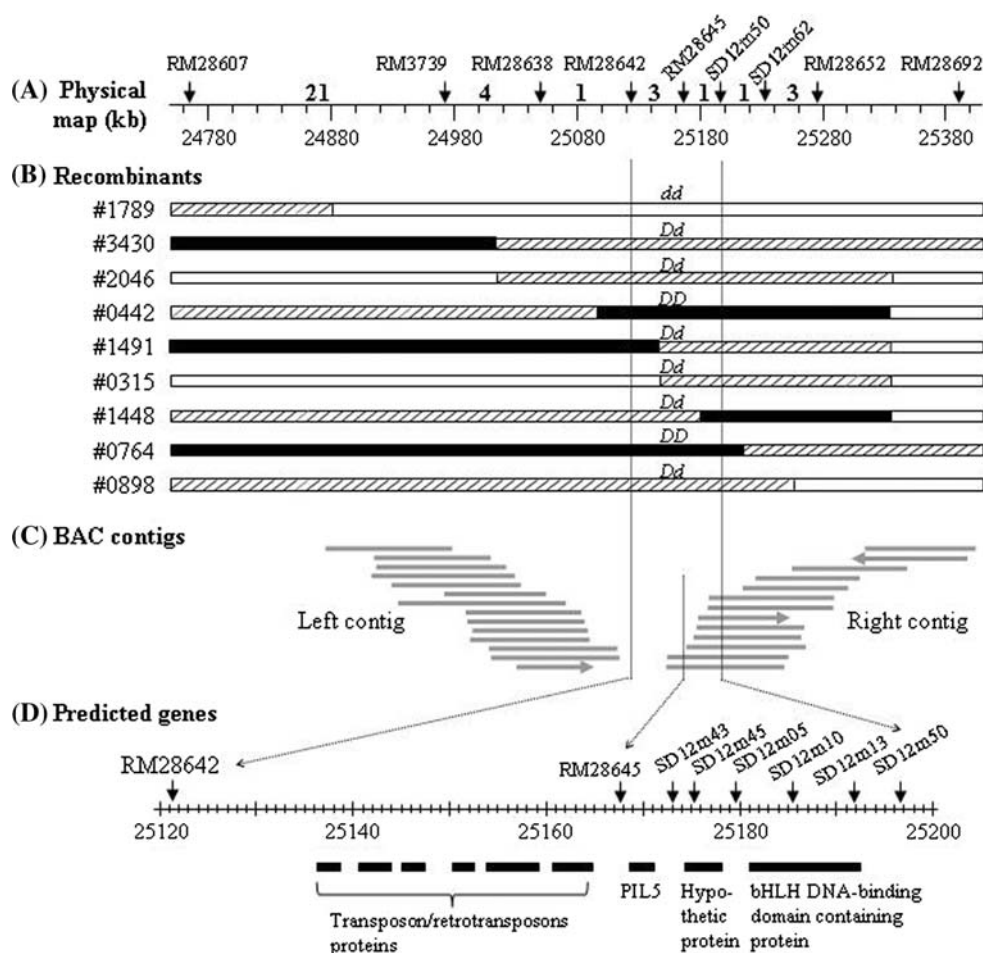
Fig. 1 The *qSD12*-containing genomic region. **a** A partial physical map for the region on rice chromosome 12. *Arrows* indicate marker positions. *Figures* indicate numbers of recombinant events between the markers identified from 7,200 gametes. **b** Graphic representation of selected recombinant genotypes. Hatched or *black bars* indicate the plant having one or two copies of the segment introduced from the donor parent SS18-2 into the background of the recipient parent EM93-1 (*empty bars*). The segment end(s) are arbitrarily defined as mid-points between adjacent markers. The *vertical lines* delimit the narrowed *qSD12* region determined by the marker-assisted progeny test (Table 1). The letter *D* or *d* indicates the dormancy or non-dormancy allele. **c** BAC contigs aligned to the region. *Horizontal bars*

indicate bacteria artificial chromosome (BAC) clones screened from the library constructed for the weedy rice line SS18-2. The contigs were developed by aligning clone end-sequences to the Nipponbare genome sequence (IRGSP 2005). An *arrow-headed line* indicates the marker-confirmed clone positioned based on one end sequence; the position of the other end (*arrow head*) is uncertain because the end sequence has multiple hits on different chromosomes with a low identity. **d** Predicted genes in the narrowed *qSD12* region. *Black bars* indicate the nine predicts based on the Nipponbare genome sequence (IRGSP 2005) available at http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice. PIL5: phytochrome-interacting factor3-like 5; bHLH: basic helix-loop-helix

were genotyped with an additional set of 6 markers distributed on the 500-kb region. Figure 1a shows distribution of the recombinant events on the seven marker intervals.

A significant marker-germination correlation was detected in the progeny lines derived from recombinant plants #3430, 2046, 1491, 0315, 1448, and 0898 (Table 2), indicating that the recombinants are heterozygous for the *qSD12* locus. Because these 6 recombinant plants share a heterozygous interval between RM28642 and SD12m50, it is concluded that the *qSD12* underlying gene locates on the shared interval of <75 kb (Fig. 1b). This dormancy gene

accounted for 28–69% of phenotypic variances under experimental conditions (Table 2).

The correlation was not significant in the progeny lines derived from recombinants #1789, 0442, and 0764. This indicates there is no dormancy gene on the heterozygous intervals, which locate outside the RM28642 to SD12m50 interval (Fig. 1b). Mean germination was much higher in the recombinant #1789-derived line (92%) than in the recombinants #0442- and 0764-derived lines (6%). This is because the plant #1789 and plants #0442 and 0764 are EM93-1- and SS18-2-like homozygous, respectively, for the RM28642 to SD12m50 region (Fig. 1b).

Table 2 Summary of statistic parameters for progeny lines derived from selected recombinants

Recombinant ID[a]	Progeny lines				
	Marker[b]	N[c]	Germination (%)[d]	r (R^2)[e]	DAR[f]
#1789	RM28607	39(F)	92.6 ± 3.1	−0.068[NS]	7
#3430	RM28652	94(GH)	30.0 ± 9.7	−0.628 (0.39)[**]	7
#2046	RM28652	108(F)	25.5 ± 19.2	−0.655 (0.43)[**]	7
#0442	RM28638	100(F)	5.9 ± 7.3	−0.004[NS]	7
			74.0 ± 7.4	0.118[NS]	21
#1491	RM28652	103(F)	19.5 ± 7.2	−0.613 (0.38)[**]	7
			78.3 ± 8.8	−0.525 (0.28)[**]	21
#0315	RM28652	120(F)	23.5 ± 10.0	−0.713 (0.51)[**]	7
			84.8 ± 7.9	−0.657 (0.43)[**]	21
#1448	RM28645	108(F)	23.6 ± 6.0	−0.828 (0.69)[**]	7
			72.0 ± 9.8	−0.706 (0.50)[**]	21
#0764	RM28652	59(GH)	6.6 ± 5.9	0.109[NS]	7
#0898	RM28645	48(GH)	17.1 ± 10.0	−0.628 (0.39)[**]	7

[a] Refer to Fig. 1b for plant genotype

[b] The locus (Fig. 1a) segregating in the line

[c] Number of plants grown under field (F) or greenhouse (GH) conditions

[d] Mean ± SE

[e] Correlation coefficient (r) between marker genotypes and germination rates. Genotypes that are homozygous for the EM93-1-derived allele, heterozygous, and homozygous for the SS18-2-derived allele are coded as 1, 2, and 3, respectively for correlation analysis. The R^2 values estimate proportion of the phenotypic variance explained by the marker. The superscripts NS or ** indicate the correlation not significant at the $P = 0.05$ or significant at the $P < 0.0001$ level

[f] Days of after-ripening prior to the germination

BAC library and contigs

A total of 27 BAC clones confirmed by PCR and end-sequence alignment were anchored to the narrowed *qSD12* and its flanking regions (Fig. 1c). Three of the 27 clones were positioned based on one end sequence and the PCR products for selected markers. This is because the other end sequence has a number of hits distributed in multiple chromosomal regions of the reference genome. The end sequences that match the reference genome sequence with 97.1 (±2.0)% identity was 379 (±168) bp on average, ranging from 193 to 781 bp. For the 24 clones with both ends aligned, the mean insertion size is 98.3(±11.6) kb, ranging from 83 to 141 kb. Thus, it is estimated that the weedy rice BAC library of 36,864 clones has a total length of 3,624 magebases (Mb), which is equivalent to 8–9 times the rice haploid genome size (400–440 Mb).

The above 27 clones form two contigs that is separated by a gap of 40.2 kb (Fig. 1c). The left contig is about 15 kb away from the left border (RM28642) of the narrowed *qSD12* region. The right contig with its 5′ end covers the left side (about 2/3) of the narrowed *qSD12*. The gap length is shorter than a half of the mean insertion size. The second hybridization experiment failed to find a single clone to fill the gap. Most likely, there are some highly repeated sequences in the 40-bp region. This hypothesis is supported by the presence of predicted repetitive genes in the *qSD12* region, as stated below.

Predicted genes in the narrowed *qSD12* region

There are nine predicted genes in the narrowed *qSD12* region based on the annotated reference genome sequence (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice). They are a cluster of six transposon or retrotransposon (T/RT) protein genes and three putative genes including a phytochrome-interacting factor3-like 5 (PIL5), a hypothetic protein, and a basic helix-loop-helix (bHLH) DNA binding domain-containing protein gene (Fig. 1d). The highly repetitive T/RT genes are located in the gap region and not included in the weedy rice library. The three putative genes are located on the 25-kb interval between RM28645 and SD12m50, which is covered by the 5′-end side of the right contig (Fig. 1d). Five new polymorphic markers (Fig. 1d; Table 1) were developed to delimit each of the three genes.

Effect of the *qSD12* locus on ABA in an isogenic background

The IL_{SD12}^{DD} and IL_{SD12}^{dd} isogenic lines were identical in all visible morphologies and flowering and maturation time (data not shown) but differed greatly in germination
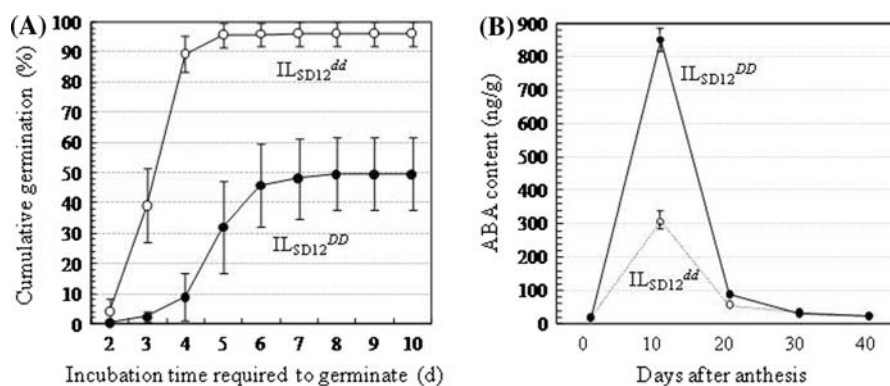
Fig. 2 Genotypic difference of *qSD12* in germination velocity seed (**a**) and seed abscisic acid (ABA) content (**b**). IL_{SD12}^{DD} and IL_{SD12}^{dd} are isogenic lines for the *qSD12* dormancy and non-dormancy alleles, respectively, selected from the recombinant #0315 (Fig. 1b)-derived population. Mean (stdev) germination values, indicated by *circles* (*bars*), were estimated for 10-day after-ripened seeds from ten plants

profile and ABA content in early developing seeds (Fig. 2). IL_{SD12}^{DD} and IL_{SD12}^{dd} had about 50 and 95% seeds germinated, respectively, after 10 days of after-ripening. Seed ABA content for IL_{SD12}^{dd} increased rapidly after fertilization to reach the highest level at about 10 days and then decreased quickly to maintain a relatively low level after 20 days (Fig. 2b). The dormant line IL_{SD12}^{DD} had little difference in ABA content from IL_{SD12}^{dd} at 0 or 20 to 40 days. The largest genotypic difference in ABA content occurred at about 10 days, when IL_{SD12}^{DD} (850 ng/g) was much higher than IL_{SD12}^{dd} (307 ng/g).

The genotypic difference in seed dormancy and ABA content between IL_{SD12}^{DD} and IL_{SD12}^{dd} was accounted for by only the *qSD12*-containing genomic region of about 200 kb, which was derived from recombinant #0315 (Fig. 1b). There is no predicted gene in the 200-kb region that has been assigned to ABA biosynthetic or metabolic pathways according to the annotated Nipponbare genome sequence (http://rice.plantbiol ogy.msu.edu/cgi-bin/gbrowse/rice). Therefore, the observed effects can be attributed to the *qSD12* underlying gene.

Discussion

Candidate genes for *qSD12*

The fine mapping experiment delimited *qSD12* to the genomic region of <75 kb with nine predicted genes (Fig. 1d). The cluster of six T/RT genes could be excluded as QTL candidates because they are highly repetitive in the rice genome and usually have little phenotypic effect. Of the remaining three genes, the predicted PIL5 (LOC_Os12g40700) or bHLH motif-containing (LOC_Os12g40730) loci are more likely candidates for the QTL underlying gene. Both PIL5 and some bHLH transcription factors are involved in regulation of seed germination in Arabidopsis mutants (Penfield et al. 2005; Finkelstein et al. 2008; Oh et al. 2009). It is noted that the

aforementioned genes were annotated based on the genome sequence of the non-dormant variety Nipponbare. Therefore, it is necessary to sequence the genomic segment from the weedy rice donor to prove the prediction.

The major effect of *qSD12* on inhibiting germination, accounting for up to 69% of the phenotypic variance (Table 2), was validated for the first time under field conditions. The relatively constant phenotypic effect makes the dormancy allele ideal for use to improve resistance to pre-harvest sprouting. Pre-harvest sprouting is a problem in some rice production areas, and for some types of rice such as male sterility lines of hybrids (Gao et al. 2008, 2009). The selected isogenic line IL_{SD12}^{DD} contains only a chromosomal segment of about 200 kb from weedy rice, on which there are 9 polymorphic markers (Fig. 1a, d). Both the plant material and fine mapping information would facilitate use of the dormancy allele in breeding programs.

The weedy rice BAC library

The BAC library constructed for the weedy rice line SS18-2 has an average insertion size of 98.3 kb and a total length equivalent to 8–9 times of the haploid genome size. The BAC clones screened from the library include those covering the best candidates of the *qSD12* underlying gene (Fig. 1c). The predicted genes in the narrowed region vary in length from a few to 12 kb (Fig. 1d). Thus, the selected clones are especially valuable for isolation of a large candidate including its flanking regions from weedy rice.

The BAC library provides a genomic tool for research on weed rice. Weedy rice is a noxious weedy in rice growing areas because many characters contribute to its adaptation to human disturbed environments or to its mimicry to cultivars (Delouche et al. 2007). Except for seed dormancy genes, QTL that have been identified from SS18-2 also include those controlling seed shattering, awn length, pigmentations on seed covering tissues, photoperiod sensitivity

and flowering time, plant height, and panicle morphologies (Gu and Foley 2007; Gu et al. 2008, unpublished data). In addition, SS18-2 was collected from Thailand (part of the rice origin center) and serves as a wild-like representative in weedy rice classification (Suh et al. 1997). Thus, the SS18-2 BAC library can be used to isolate the QTL underlying genes for almost all weedy characters and to identify genomic structure changes in relation to the evolution of cultivated or weedy rice.

Relation among *qSD12*, ABA, and seed dormancy

Previous research has associated *qSD12* with seed dormancy (Gu et al. 2004, 2005, 2006, 2008). The present research based on a pair of well-defined isogenic lines detected the effect of *qSD12* on ABA and clearly associated the hormone with seed dormancy (Fig. 2b). A relatively high level of ABA was detected in the 10-day developing seeds from IL_{SD12}^{dd}, indicating that there is a default system in the genetic background to synthesize the hormone, and the natural mutation at *qSD12* does not prevent the system from the biosynthesis. An even higher level of ABA accumulated in IL_{SD12}^{DD} developing seeds indicates that the QTL underlying gene enhances or blocks the hormone biosynthesis or degradation. It is commonly considered that ABA induces seed dormancy (Finch-Savage and Leubner-Metzger 2006; Finkelstein et al. 2008; Holdsworth et al. 2008). Therefore, it is concluded that the *qSD12* underlying gene promotes ABA accumulation in early developing seeds and then the accumulated ABA induces primary dormancy, which is characterized by delayed germination or reduced germination rate.

To our knowledge, *qSD12* is the first dormancy QTL that has been assigned a functional relationship with ABA, a positive/negative regulator of dormancy/germination. Induction of primary dormancy by ABA appeared to occur before 20 days after anthesis, because the hormone dramatically decreased to a relatively low level, and the difference in ABA content between the isogenic lines disappeared after 20 days (Fig. 2b). There was no direct correlation of ABA content with the dormancy status of dry or imbibed seeds in red weedy rice (Gianinetti and Vernieri 2007). The *qSD12* locus controls seed dormancy through the embryo and/or endosperm tissues (Gu et al. 2008). These results suggest that a high level of ABA initially synthesized in seed offspring tissue is critical to induce and also likely to maintain seed dormancy.

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